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Studies on the Constituents of Higher Fungi of Korea(XX)

Amino Acids and Lipids from *Strobilomyces floccopus* and *Coprinus comatus*

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Abstract□ To investigate constituents of *Strobilomyces floccopus* (Fr.) Karst. and *Coprinus comatus* (Fr.) S.F. Gray, free and total amino acids of the two mushrooms were quantitatively analyzed by G.L.C. and an amino acid analyzer. Free amino acids were extracted from both mushrooms with ethanol. Fourteen free amino acids were detected from the ethanol extract of *S. floccopus* and fifteen free amino acids from *C. comatus* by G.L.C. And the dry carphopores of both mushrooms were hydrolyzed with hydrochloric acid and then the total protein amino acids were analyzed by A.A.A. Seventeen total amino acids were detected from each acid-hydrolysate of *S. floccopus* and *C. comatus*. Lipids were extracted from the carpophores of *S. floccopus* and saponified with alcoholic potassium hydroxide. The isolated sterols were subjected to G.L.C. and two sterols were detected. The isolated free fatty acids were methylated with diazomethane and subjected to column chromatography and G.L.C. Eleven saturated and nine unsaturated free fatty acids were detected from the carpophores of *S. floccopus*. The presence of these nutrient components shows that the two mushrooms can be utilized as edible ones.

Keywords□ Basidiomycetes—Agaricales—*Coprinus comatus* (Fr.) S.F. Gray and *Strobilomyces floccopus* (Fr.) Karst. Free and total amino acids—saturated and unsaturated fatty acids—stigmasterol and β-sitosterol.

More than 600 species of Korean mushrooms have been identified up to 1978. In studies of constituents of Korean higher fungi, Kim[1] reported the identification of amino acids in 15 edible mushrooms in 1958. Yoon[2] reported that the extracts of 33 species among 81 Korean mushrooms had antibiotic activities in 1959. Huh reported on the amino acids of 27 species of Korean edible mushrooms in 1960, and Kim *et al.*[3] reported on the detection of alkaloids of Korean mushrooms in 1971. Recently, Park *et al.*[4] reported that three species of Korean mushrooms had antitumor activities in 1978.

There have been several reports on *Coprinus comatus* (Fr.) S.F. Gray (family *Coprinaceae*), an edible mushroom. Lipids,[5~7], proteins[8], amino acids[9], trace elements[10] and several enzymes[11] of *C. comatus* were studied. The autolysis of its sporophores[12], its carbon metabolism in submerged culture[13] and proteloytic activity of *C. comatus*[14] were also reported. Recently disulfiram-like actions of *Coprinus* species[15] were reported.

Strobilomyces floccopus (Fr.) Karst. (family *Strobilomycetaceae*) is an edible mushroom. There are a few reports on *S. floccopus*, cancer inhibitor[16] from, basidiospore germination[17] by and dihydroxy phenylalanin from wound tissue of it[18]. But studies on the basic constituents of *S. floccopus* have not been re-

ported.

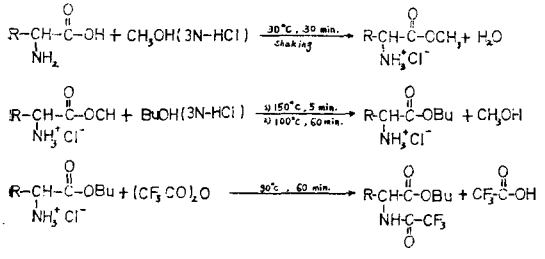
Therefore the authors attemped to investigate amino acids and lipids of *S. floccopus* and those of *C. comatus*.

EXPERIMENTAL

Materials

The carpophores of *Coprinus comatus* (Fr.) S.F. Gray (family *Coprinaceae)* were collected in the area of Galmae-Ri, Gyung Gi-Province during the period from September to October, 1978. The carpophores of *Strobilomyces floccopus* (Fr.) Karst. (family *Strobilomycetaceae*) were collected in the Arboretum of College of Agriculture, Seoul National University at Suwon during the period from June to July, 1978.

Both mushrooms were dried in open shadow for several hours and then in an oven at 50°C for six hours. The carpophores of *C. comatus* and *S. floccopus* were preserved as voucher



Scheme I: Synthesis of TFA-butyl ester

specimens at the Department of Microbial Chemistry, College of Pharmacy, Seoul National University.

Analysis of Amino Acids

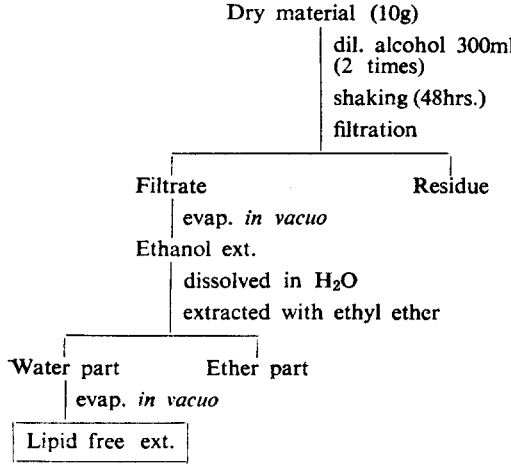
1) Free Amino Acids by G.C.[19]

Reagents used were as follows. *n*-Butanol (HCl) : made by saturation of dry HCl gas in 100 ml of *n*-butanol. Methanol(HCl) : made by saturation of dry HCl gas in 100 ml of anhydrous methanol. Methylene chloride (anhydrous) : made by reflux of 100 ml of reagent grade CH_2Cl_2 over 25 g of anhydrous $CaCl_2$ for 30 minutes, distilled in all-glass apparatus and stored in an all-glass inverted top bottle to protect from atmospheric moisture. Trifluoroacetic anhydride (TFAA) was purchased from E. Merck AG, Darmstadt.

Synthesis of derivatives of standard amino acids(Scheme I) was carried out as follows. Three ml of CH_3OH(HCl) were added to the standard amino acids mixture containing 16 protein amino acids standard at individual weights of 1.5 mg. The solution of standard amino acids mixture was esterified for 30 minutes at room temperature and then it was evaporated to dryness. Three ml of butanol(HCl) were added to these methyl esters and then esterified for five minutes at 150°C on an oil bath and for 60 minutes at 100°C on a water bath. Then this solution was evaporated to dryness. Three ml of anhydrous CH_2Cl_2 and one ml of TFAA were added to these butyl esters and then acylated for 60 minutes at 90°C. Then this solution was evaporated to dryness and the residue was dissolved in $CHCl_3$(Merck) to the volume of one ml. Each of 16 standard amino acids at individual weights of 1.5 mg was under the same procedure as above.

Extraction of free amino acids(Scheme II) was conducted as follows. Three-hundred

ml of ethanol were added to 10 g of a dried material and homogenized with a Waring blendor. Then extraction was conducted on an orbital shaker at 30±1°C for 48 hours. After filtration, 250 ml of ethanol were added to the filtration residue and the second extraction was carried out under the same condition. After filtration again, the filtrate was evaporated to dryness, and the weight of the ethanol extracts of *C. comatus* was 2.395 g and that of *S. floccopus* was 1.245 g. These ethanol extracts were dissolved in water, and from them, lipids were removed with ethyl ether. Then the water layer was evaporated to dryness, and the weight of the lipid-free extracts of each was 2.021 g of *C. comatus* and 1.233 g of *S. floccopus*.



Scheme II: Extraction procedure (G.C.)

Synthesis of derivatives of lipid-free extracts from *C. comatus* and *S. floccopus* was carried out. Considerable amounts of anhydrous CH_2Cl_2 were added to a sample of 52.1 mg of *C. comatus* and a sample of 27.5 mg of *S. floccopus* from the prepared lipid free extracts. These were evaporated to dryness and analyzed in the same procedure as the standard amino acids mixture.

The instrument used for this analysis was a Shimazu Gas Chromatograph(Model G.C.-4BM) and the measurement conditions are shown in Table I.

Table I: Measurement conditions(G.C.)

Column	3% OV-17 (80–100 mesh shimalite) 3 mm ø × 2 m boronsilicate glass column	
Detector	Flame Ionization Detector	
Temperature	Injection port	250°C
	Column	100–210°C (5°C/min.)
	Detector	270 C
Flow rate	N_2	40 ml/min.
	H_2	60 ml/min.
	Air	80 ml/min.
Attenuation	4×10^2	a.f.s. (ampere full scale)

Quantitative analyses of free amino acids were carried out by the triangulation procedure.

2) Total Amino Acids by A.A.A.

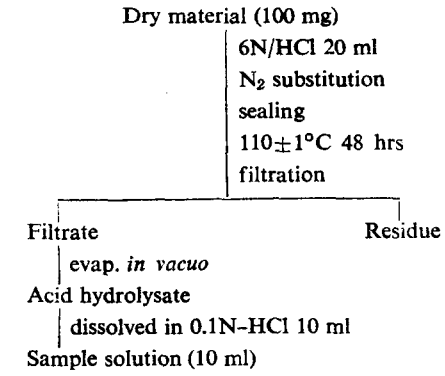
Each of the 17 standard amino acids was diluted, with pH 2.2 citrate buffer, to the concentration of 0.2 μM/ml(14 standard amino acids), and of 0.1584 μM/ml of glutamic acid, 0.4 μM/ml of proline and 0.1906 μM/ml of cysteine. Sample solutions (Scheme III) were prepared as follows.

Twenty ml of 6N-HCl were added to 100mg of the dried material in an ampule of 100 ml volume. After nitrogen gas substitution and sealing of the ampule, the sample was hydrolyzed for 48 hrs. at 110±1°C in an oven. After the filtration, the filtrate was evaporated to dryness.

This acid hydrolysate was dissolved in 10 ml of 0.1N-HCl and diluted to an appropriate

concentration with pH 2.2 citrate buffer for the instrumental measurement of total amino acids.

The instrument used for this analysis was a Hitachi amino acid autoanalyzer(Model KLA-5) and the measurement conditions



Scheme III: Sampling procedure (A.A.A.)

Table II: Measurement conditions(A.A.A.)

Column size	9 mm ID × 550 mm	
	9 mm ID × 100 mm	
Ion exchange resin	Hitachi-custom ion exchange 2163	
	Hitachi-custom ion exchange 2611	
Flow rate	Buffer solution:	60 ml/hr
	Ninhydrin:	30 ml/hr
Wave length	15 mm tublar flow cell, 570 nm(red). 440 nm (green)	
Buffer solution	pH 3.25, pH 4.25, pH 5.28 Sodium citrate buffer solution	
Column temperature		55°C
Reaction bath temperature		100°C

are shown in Table II.

Quantitative analyses of total amino acids were conducted by the HW method.

Analysis of Free Fatty Acids and Sterols

1) Extraction and Purification of Lipids

Lipids from 20 g of dry material of *S. floccopus* and 40 g of wet material of *C. comatus* were extracted two times with $CHCl_3$: MeOH (2:1) solvent mixture on an orbital shaker at room temperature. And purified lipids were obtained by removal of non-lipid substance[20] after removal of denatured proteolipids[21] from the extracts.

2) Saponification of Lipids

These purified lipids were saponified with 10 % ethanolic KOH for four hours at 80°C on a water bath under nitrogen gas. Then each of the saponified and the unsaponified fractions was separated.

3) Isolation and Identification of Sterols

Sterol fractions were isolated from the unsaponified fraction by the use of preparative TLC[22], and each fraction positive to Liebermann-Bürchard test was applied to gas chromatography with four authentic sterols.

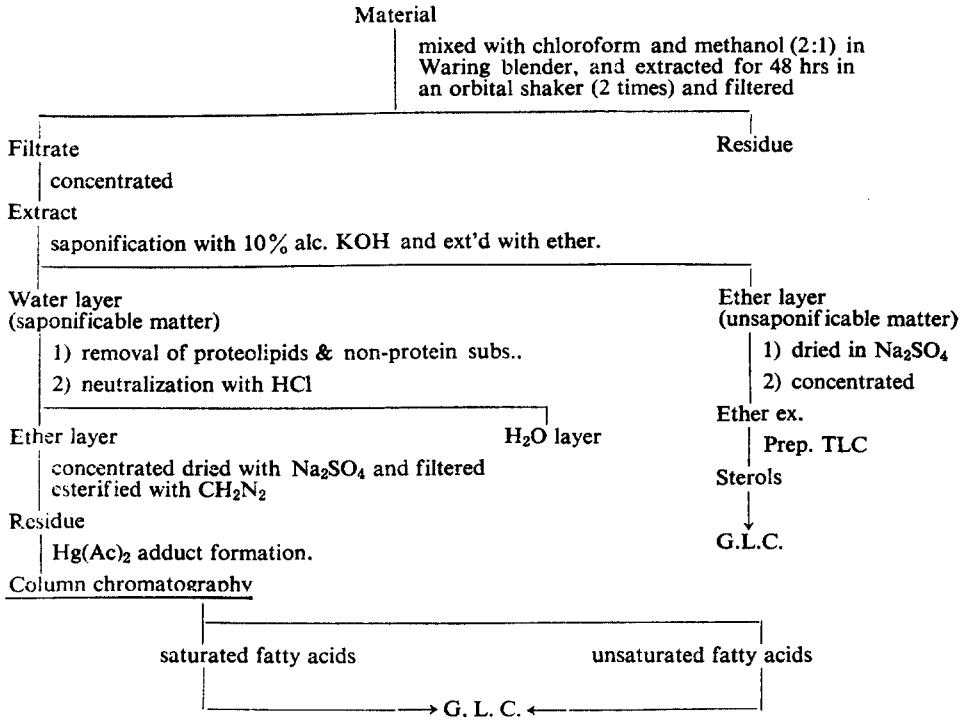
The gas chromatogram of each material was made and the identification of sterols were made in comparison with standard calibration curves[23].

4) Instrument and Measurement Conditions

The instrument used for this analysis was a Shimazu Gas Chromatograph (Model GC-4BM) and the measurement conditions are shown in Table III.

5) Isolation of Free Fatty Acids and Preparation of Methyl Esters

The water layer obtained by the saponification of dry materials of *S. floccopus* was neutralized with 10 % HCl and the resulting free fatty acids were extracted with ethyl ether. The prepared free fatty acids were methylated with diazomethane in the use of



Scheme IV: Analysis of lipids.

N-nitrosomethylurea and preserved for 15 hours at 4°C. Then the identification of methylation was conducted by TLC.

6) Separation of Saturated and Unsaturated Fatty Acids Methyl Esters[24]

Synthesis of $Hg(Ac)_2$ Adduct: A sample of 30 mg of dry methyl esters of the fatty acids and a portion of 150 mg of mercuric acetate were placed in a culture tube(16 × 150 mm) equipped with a teflon-lined screw cap and then 10 ml of a solution containing 5 % dist. water and 0.3 % glacial acetic acid in absolute methanol was added. The resulting tightly-sealed tube was heated on a water bath at 60°C for approximately 5 min. To ensure solution of the mercuric acetate, the tube was stored in the dark at room temperature for 24 hours. The solvent and excess acetic acid were removed and the residue was dried by evaporation under nitrogen gas at room temperature. The dry residue was shaken several times with 15 ml benzene at 50–60°C,

Table III: Measurement conditions(G.L.C.)

Column	3% OV-17 (80–100 mesh shimalite)	
	3 mm ø × 2 m boronsilicate glass column	
Detector	Flame Ionization Detector	
Temperature	Injection port	250°C
	Column	270°C
	Detector	280°C
Flow rate	N_2	60 ml/min.
	H^2	70 ml/min.
	Air	40 ml/min.
Attenuation	4 × 10_2	a.f.s. (ampere full scale)

and the extracts were filtered through glass-wool into a column of silica gel.

Column Chromatography: The silica gel column was prepared from a slurry in benzene which was poured into pasteur pipet(250 mm in height). The column was eluted with the benzene to a total volume of 300 ml. This eluate contained the methyl esters of saturated fatty acid mercuric acetate adducts remaining at the top of the column as indicated by a yellow band. The benzene eluate was evaporated to dryness under nitrogen gas.

The mercuric acetate adducts were eluted with 150 ml of 5 % glacial acetic acid in absolute ethanol. To recover the methyl esters of the unsaturated fatty acids, this eluate was treated with 30 ml of 6N hydrochloric acid and 150 ml of water, after five min. This mixture was diluted with 150 ml water and extracted with 40 ml of benzene. The resulting benzene fractions were dried with anhydrous sodium sulfate and evaporated to dryness under nitrogen gas. This dry residue was dissolved in $CHCl_3$(Merck grade) and then applied to G.C. with four authentic saturated and two authentic unsaturated fatty acid methyl esters.

7) Instrument and Measurement Conditions
The instrument used for this analysis was a Pye Unicam GCV Chromatograph and the measurement conditions are shown in Table IV.

8) Identification of Fatty Acids Methyl Esters

The semilog plot of the log of the retention time versus the carbon number of a homologous series was linear (Fig. 1) and this fact was established for the identification of various components described by Miwa[25] and Ruseva-Atanasona[26].

RESULTS

Analysis of Free Amino Acids(G.C.)

The gas chromatogram of standard amino acids(Fig. 2) was obtained by using one μl of 14 standard amino acids mixture N-TFA butyl esters and each gas chromatogram of *Strobilomyces floccopus* and *Coprinus comatus*

Table IV: Measurement conditions(G.L.C.)

Column	5% DEGS, 1% H_3PO_4 on 80–100 mesh shimalite	
	5 mm ø × 2 m boron silicate helix shaped	
Detector	Flame Ionization Detector	
Temperature	Injection port	200°C
	Column	185°C
	Detector	230°C
Flow rate	N_2	70 ml/min.
	H_2	60 ml/min.
	Air	40 ml/min.
Attenuation	16×10^3	a.f.s.

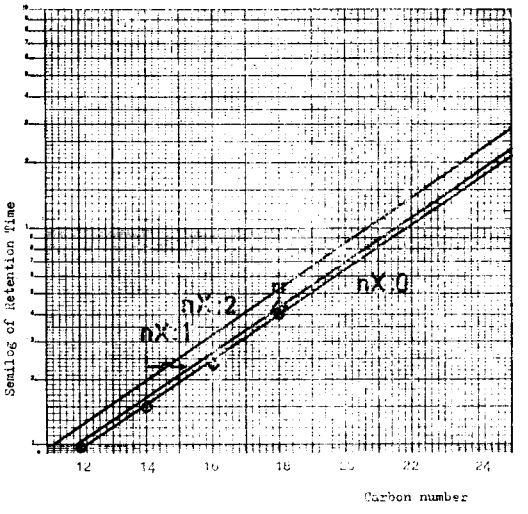


Fig. 1: Retention times of methyl esters of standard fatty acids

Table V: Contents of free amino acids (mg/g)

Amino Acid	Strobilomyces floccopus	Coprinus comatus
Ala	5.54	1.31
Thr	3.67	5.59
Gly	4.16	1.44
Ser	1.00	0.41
Val	3.97	20.29
Ile+Leu	0.52	1.74
His	2.82	73.00
Pro	21.95	9.05
Met	10.17	3.14
Asp	13.98	—
Phe	0.07	9.56
Tyr	—	3.85
Lys+Glu	9.78	4.75
Trp	—	2.75

(Figs. 3, and 4) was obtained by using 1 μl of each sample solution. The contents of free amino acids were shown in Table V.

Analysis of Total Amino Acids(A.A.A.)

The chromatogram of standard amino acids(Fig. 5) was obtained by using 0.5 ml of standard amino acids mixture and the chromatograms of *S. floccopus* and *C. comatus*

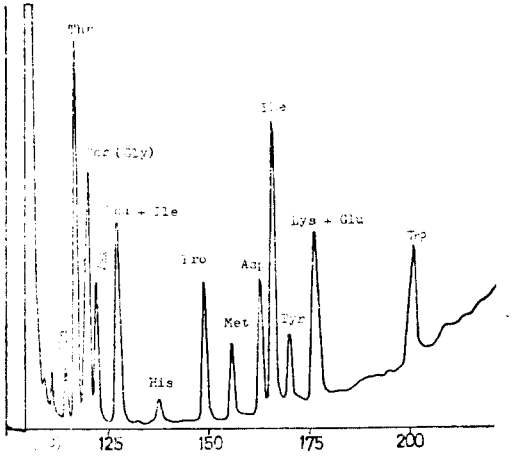


Fig 2: Chromatogram of standard amino acids(G.C.)

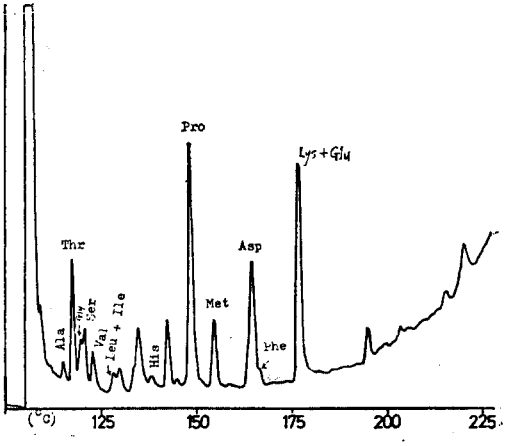


Fig 3: Chromatogram of amino acids of *Strobilomyces floccopus*(G.C.)

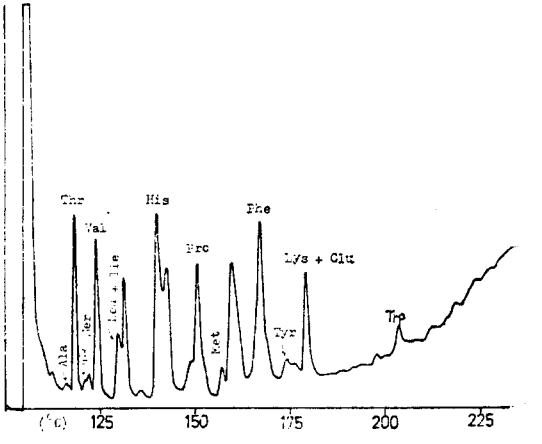


Fig 4: Chromatogram of amino acids of *Coprinus comatus*(G.C.)

(Fig. 6,7) were btaoined by using 0.5 ml of each sample solution. The contents of total amino acids were shown in Table VI.

Analysis of Lipids

1) Identification of Sterols

The gas chromatogram of sterol fractions

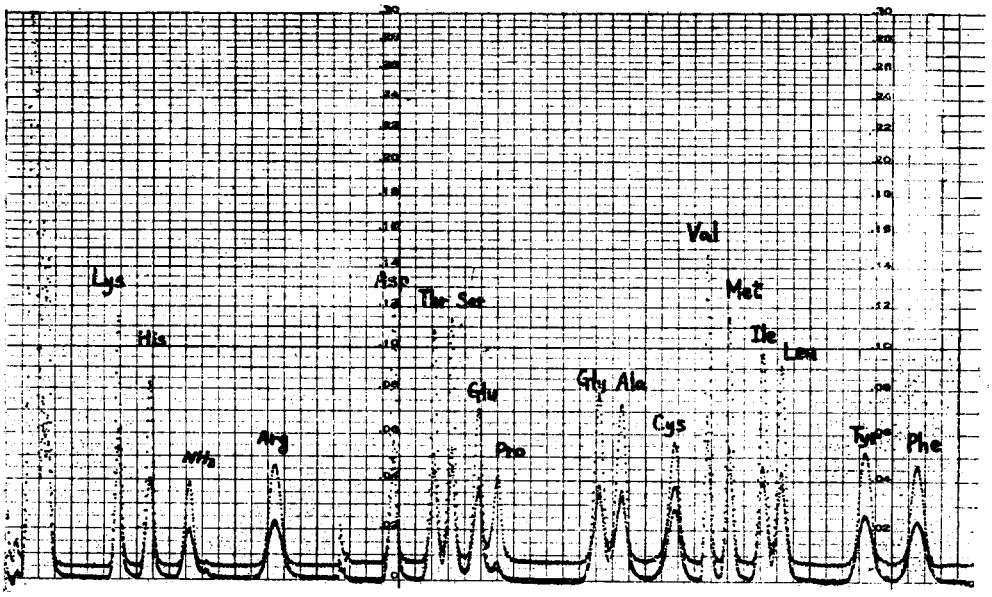


Fig 5: Chromatogram of standard amino acids (A.A.A.)

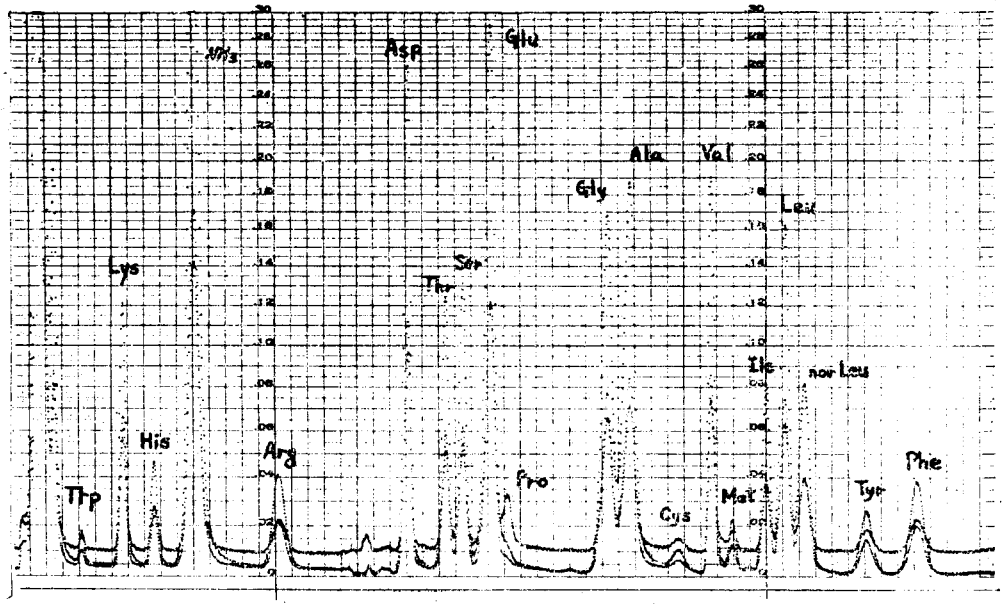


Fig. 6: Chromatogram of amino acids of *Strobilomyces floccopus* (A.A.A.)

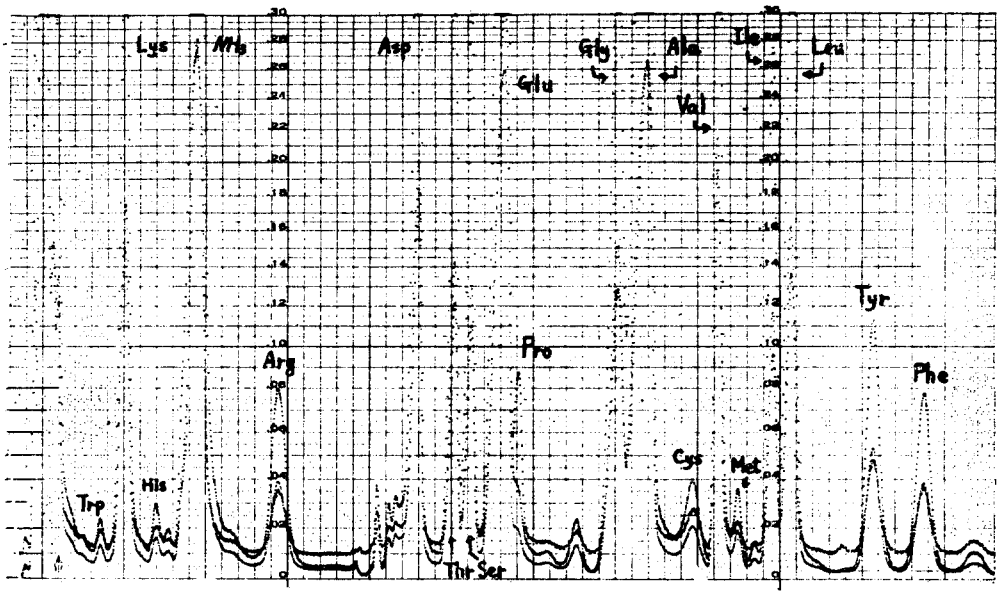


Fig. 7: Chromatogram of amino acids of *Coprinus comatus* (A.A.A.)

Table VI: Contents of total amino acids(mg/g)

Sample Amino Acid	Strobilomyces floccopus	Coprinus comatus
Lys	3.50	7.50
His	1.70	0.58
Arg	3.34	6.84
Asp	5.68	12.00
Thr	25.80	6.98
Ser	25.40	4.66
Glu	10.74	34.50
Pro	2.34	5.28
Gly	3.22	7.52
Ala	4.26	21.50
Cys	0.42	1.18
Val	3.22	12.50
Met	0.60	0.82
Ile	1.96	5.56
Leu	4.06	12.00
Tyr	1.96	8.02
Phe	2.62	4.94
TOTAL	100.82	152.38

in *S. floccopus* (Fig. 8) was obtained by analyzing each sample solution.

2) Identification of Free Fatty Acids

The gas chromatograms of fatty acid methyl esters in *S. floccopus*(Figs. 9 and 10) were obtained by analyzing each solution of saturated and unsaturated fatty acid methyl esters. Eleven peaks of the saturated fatty acid methyl esters, nine peaks of the unsaturated fatty acid methyl esters and three peaks unidentified fatty acid methyl esters were detected on the gas chromatograms.

DISCUSSION

In the analyses of amino acids contained in *Strobilomyces floccopus* and *Coprinus comatus*, as mentioned above, sixteen free amino acids were identified and quantitated. Isoleucine and leucine, lysine and glutamic acid were quantitated simultaneously because their peaks were concurrent at the same

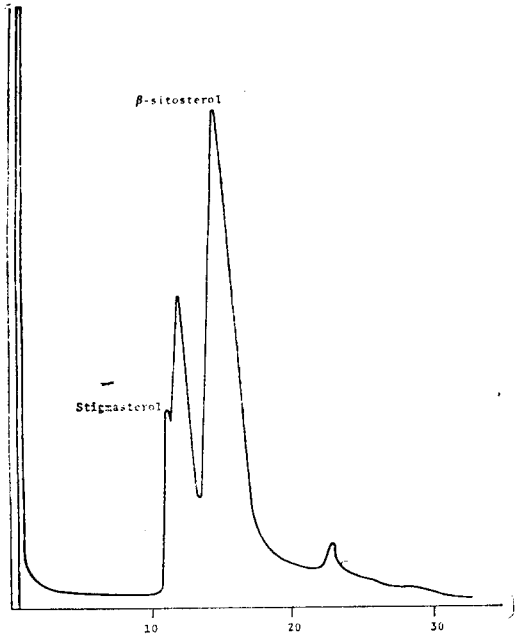


Fig. 8. Gas chromatogram of sterol fractions of *S. floccopus*

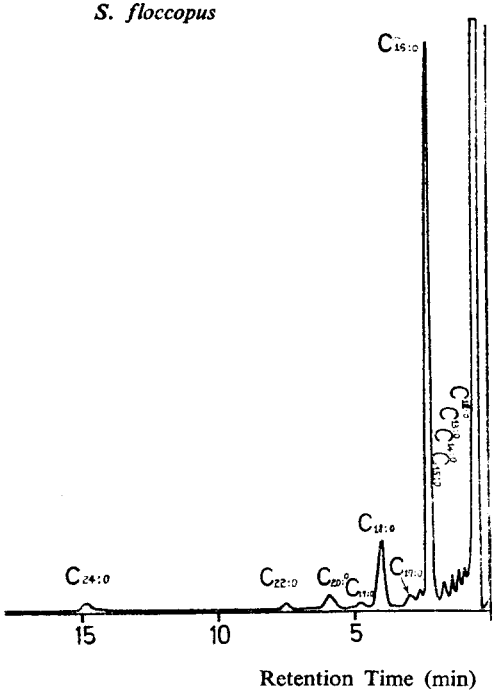


Fig. 9. Gas chromatogram of saturated fatty acid from *S. floccopus*

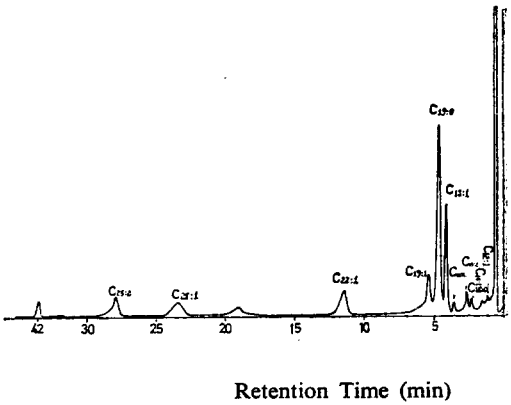


Fig. 10: Gas chromatogram of unsaturated fatty acid methyl esters from *S. floccopus*

retention times on the gas chromatograms (Figs. 2, 3 and 4). There were also several unidentified peaks on the gas chromatograms. It is presumed that *S. floccopus* and *C. comatus* may contain several unusual amino acids.

And in the total amino acid analysis, tryptophan was mostly destructed during the procedure of acid hydrolysis and therefore it was not quantitable. It was reported that 5.3 % of threonine and 10.5 % of serine were decomposed during acid(6N-HCl) hydrolysis for 24 hours at 100°C and that approximately 5 % of threonine, cysteine, tyrosine and 10 % of serine were destructed when hydrolyzed for 22 hours[28].

The total amounts of total amino acids in *S. floccopus* and *C. comatus* were 100.82 mg/g and 152.38 mg/g, respectively. In the analysis of free fatty acids in *S. floccopus*, diazomethane gas was prepared by locating N-nitrosomethylurea powder on the border line of ethyl ether and 10 % KOH solution[29].

CONCLUSION

It was shown that the carpophores of *Strobilomyces floccopus* contain 14 free amino acids : alanine, glycine, valine, leucine, isoleucine, threonine, serine, aspartic acid, glutamic acid, methionine, histidine, lysine, proline and phenylalanine. And the carpophores of *Coprinus comatus* contain 15 free amino acids : alanine, glycine, valine, leucine, isoleucine, threonine, serine, glutamic acid, methionine, histidine, lysine, proline, phenylalanine, tyrosine and tryptophan. Each of the carpophores of *S. floccopus* and *C. comatus* contains 17 total amino acids.

From the sterol fraction of *S. floccopus*, two sterols, stigmasterol and beta-sitosterol, were identified.

From the free fatty acid fraction of *S. floccopus*, eleven saturated fatty acids and nine unsaturated fatty acids were identified. Among the nine unsaturated fatty acids, there were seven monoenoic acids and two dienoic acids. Fatty acids of odd carbon number were found to be four in the saturated fatty acid fraction and five in the unsaturated fatty acid fraction. The existence of these nutrient constituents indicates that the two mushrooms can be utilized as edible ones.

ACKNOWLEDGMENTS

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